PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 4 : C12P 19/04, C08B 37/00 A61K 31/715	A1	(11) International Publication Number: WO 86/ 04355 (43) International Publication Date: 31 July 1986 (31.07.86)

(21) International Application Number: PCT/US86/00066

(22) International Filing Date: 16 January 1986 (16.01.86)

(31) Priority Application Numbers: 692,692 815,957

(32) Priority Dates: 18 January 1985 (18.01.85) 9 January 1986 (09.01.86)

(33) Priority Country: US

(71) Applicant: BIO-TECHNOLOGY GENERAL CORP. [US/US]; 375 Park Avenue, New York, NY 10152 (US).

(72) Inventors: NIMROD, Abraham ; 8 Eisenberg Street, Rehovot (IL). GREENMAN, Benjamin ; 6 Hadar Street, Rehovot (IL). KANNER, Dov ; 21 Gordon Street, Rehovot (IL). LANDSBERG, Moshe ; 2 Zahal Street, Petah Tikva (IL).

(74) Agent: WHITE, John, P.; Cooper, Dunham, Clark, Griffin & Moran, 30 Rockefeller Plaza, New York, NY 10112 (US).

(81) Designated States: AT (European patent), AU, BE (European patent), BG, BR, CH (European patent), DE (European patent), DK, FI, FR (European patent), GB (European patent), HU, IT (European patent), JP, KR, LU (European patent), NL (European patent), NO, RO, SE (European patent).

Published
With international search report.

(54) Title: METHOD OF PRODUCING HIGH MOLECULAR WEIGHT SODIUM HYALURONATE BY FERMENTATION OF *STREPTOCOCCUS*

(57) Abstract

A novel mutant microorganism *Streptococcus zooepidemicus* HA-116 ATCC 39920. The microorganism produces large amounts of high molecular weight hyaluronic acid. The invention provides a method of obtaining such microorganisms. The invention also concerns a method of obtaining sodium hyaluronate which comprises growing with vigorous agitation a microorganism of the genus *Streptococcus* under appropriate conditions in a suitable nutrient medium containing a sugar component as a carbon source. The sugar component is present in a substantially constant concentration between 0.2 and 10 grams per liter. The medium has a substantially constant pH between about 6.0 and 7.5 and includes a substantially constant magnesium ion concentration above 0.05 grams per liter. The sodium hyaluronate excreted into the medium by the organism is purified using methods involving precipation, redissolving and reprecipating the hyaluronate. Composition of sodium hyaluronate which are characterized by an absence of pyrogenicity and skin irritation are obtained.

METHOD OF PRODUCING HIGH MOLECULAR WEIGHT SODIUM HYALURONATE BY FERMENTATION OF STREPTOCOCCUS

This application is a continuation-in-part of U.S. Serial No. 692,692, filed January 18, 1985, the contents of which are hereby incorporated by reference into the present application.

BACKGROUND OF THE INVENTION

This invention concerns a process for the production of the sodium salt of high molecular weight hyaluronic acid by large-scale fermentation of a microorganism of the genus *Streptococcus*.

Hyaluronic acid is a naturally occurring glycosaminoglycan consisting of a linear polymer of molecular weight of 50,000-13,000,000 daltons. It is a polysaccharide made of a repeating units of glucuronic acid and N-acetyl-glucosamine, bound by alternating 1-3 and 1-4 bonds.

Hyaluronic acid is present in various connective tissues of animals, such as skin and cartilage. Some organs are specifically rich in hyaluronic acid, such as the umbilical cord, synovial fluid, the vitreous humor and rooster combs. In addition, hyaluronic acid is produced by various microorganisms, such as streptococci Type A and C.

In skin and cartilage, the role of hyaluronic acid is to bind water and retain the tonicity and elasticity of the tissue. In joint fluids, the viscous hyaluronic acid solution serves as a lubricant to provide a protective environment to the cells. A solution of ultra-

84, 162 (1976). These procedures included anaerobic fermentations of the pathogenic bacteria, and resulted in yields of 0.4-1 grams/liter of hyaluronic acid of a molecular weight of 700,000 or less.

Other procedures have concerned the aerobic fermentation of streptococci to produce hyaluronic acid such as Japanese Patent Publication Kokai No. 58-056692, published April 4, 1983, by inventors, Akasaka H, et al. Other publications such as, U.S. Patent No. 4,141,973, February 27, 1979 by E.A. Balazs, concerned the production and purification of hyaluronic acid from sources such as animal connective tissue. The hyaluronic acid production and purification procedures disclosed in the prior art did not, however, yield hyaluronic acid of an average molecular weight of greater than $2.0x10^6$ daltons. This is largely due to the fact that hyaluronic acid is easily degraded by shearing or oxidized in reactions catalyzed by impurities or metal ions present in the hyaluronic acid composition.

The novel process described herein results in hyaluronic acid of a molecular weight from about $1x10^6$ to about $4.0x10^6$ daltons, in a yield of about 2 grams/liter in anaerobic fermentation and about 4-6 grams/liter in aerobic fermentation. This was made possible by producing a mutant strain of a Type C Streptococcus zooepidemicus, HA-116, ATCC 39920, which is a high producer of hyaluronic acid and is haemolysin minus, i.e. of negligible pathogenicity. Aerobic Fermentation of S. zooepidemicus, HA-116, ATCC 39920 and subsequent purification of hyaluronate have resulted in batches of sodium hyaluronate with an average molecular weight of greater than $3.5x10^6$ daltons. This

Summary of the Invention

The invention concerns a microorganism of the species *Streptococcus zooepidemicus*, HA-116, ATCC 39920, and mutants derived therefrom which are capable of producing sodium hyaluronate by fermentation and excreting it into the surrounding medium.

The invention also concerns a method of obtaining sodium hyaluronate which comprises growing with vigorous agitation a microorganism of the genus *Streptococcus* under appropriate conditions in a suitable nutrient medium. The medium includes a sugar component as the carbon source in a substantially constant concentration between about 0.2 and 10 grams per liter, has a substantially constant pH between about 6.5 and 7.5 and also includes a substantially constant magnesium ion concentration above 0.05 gram per liter. The microorganism produces sodium hyaluronate and excretes it into the medium. The sodium hyaluronate is then recovered from the medium.

The sodium hyaluronate is recovered from the medium by a method comprising treating the medium containing the microorganism so as to remove the microorganism and other materials insoluble in the medium, precipitating the sodium hyaluronate from the medium, e.g. precipitation with organic solvents, and recovering the precipitate. The precipitate can then be ground and dried. Compositions of sodium hyaluronate characterized by an absence of pyrogenicity and inflammatory activity can be produced by these methods.

The present invention also concerns a method for selecting microorganisms which produce enhanced amounts

Detailed Description of the Invention

The present invention concerns a method of obtaining high molecular weight sodium hyaluronate from microorganisms of the genus *Streptococcus*, e.g. *S. zooepidemicus* or *S. equisimilis*. The method comprises growing microorganisms of the genus Streptococcus with vigorous agitation under appropriate conditions and in a suitable nutrient medium. The medium includes a sugar component as the carbon source in a substantially constant concentration between about 0.2 and 10 grams per liter, a substantially constant magnesium ion concentration above about 0.05 grams per liter and a substantially constant pH between about 6.5 and 7.5. The microorganisms produce sodium hyaluronate and excrete it into the medium. The sodium hyaluronate is then recovered from the medium.

Any hyaluronic acid producing species of *Streptococcus* can be used in practicing this invention, e.g. *S. zooepidemicus*, *S. equisimilis* or *S. pyogenes*. The preferred species is *S. zooepidemicus* and the strain is *S. zooepidemicus* HA-116 ATCC 39920 which is a mutant strain produced according to a method of this invention for obtaining microorganisms which produce an enhanced amount of hyaluronic acid.

The sodium hyaluronate can be obtained by growing the *Streptococcus* under aerobic or anaerobic conditions. In a preferred embodiment of the invention the appropriate growing conditions comprise aeration of the medium at a rate greater than about 0.5 volumes of air per volume of medium per minute (vvm). An aeration rate of 1-2 vvm is generally used, however greater aeration rates may be desirable. In this preferred

a pr cipitate of sodium hyaluronate. The first, second and third organic solvents can each be isopropanol, ethanol or acetone. Alternatively the hyaluronate can be precipated by the same organic solvent in each step, e.g. sodium hyaluronate is precipitated from the medium by using isopropanol in all three of the precipitation steps.

In another embodiment of the invention the pH of the medium containing the microorganism is adjusted to about 7.0 and the medium is cooled to a temperature between about 4° and 15°C and preferably between about 4° and 20°C, prior to treating the medium to remove the microorganism. The medium is then diluted with 3% aqueous sodium acetate to the extent necessary to permit subsequent treatment e.g. three to four-fold.

In one embodiment of the invention, the sodium hyaluronate precipitate is redissolved in 0.15M aqueous NaCl and cetyl-pyridinium chloride is added to form the cetyl-pyridinium salt of hyaluronic acid. The cetyl-pyridinium salt is dissolved in aqueous NaCl and 15% ethanol, e.g. at least 1M NaCl and sodium hyaluronate is recovered therefrom by addition of organic solvent e.g. ethanol precipitating the sodium hyaluronate.

This sodium hyaluronate precipitate can be redissolved in 0.15M aqueous NaCl. Cetyl-pyridinium chloride is added to again form the cetyl-pyridinum salt of hyaluronic acid. The hyaluronic acid salt is dissolved in NaCl (at least about 1M) and ethanol and the sodium hyaluronate is recovered by addition of organic solvent. The precipitate is thereafter dissolved in sterile aqueous 1M NaCl and the resulting solution is contacted with a magnesium silicate absorbant, e.g. Flori-

solved in aqueous NaCl containing 10% (v/v) ethanol, e.g. at least 1M NaCl, and sodium hyaluronate is recovered therefrom by addition of organic solvent, e.g. ethanol.

This sodium hyaluronate precipitate can be redissolved in 0.15M aqueous NaCl. Cetyl-pyridinium chloride is added to again form the cetyl-pyridinium salt of hyaluronic acid. The hyaluronic acid salt is dissolved in NaCl (at least about 1M) with 10% ethanol and the sodium hyaluronate is recovered by addition of organic solvent. The precipitate is thereafter dissolved in sterile aqueous 1M NaCl and the resulting solution is contacted with a magnesium silicate absorbant, e.g. Florisil to remove impurities and residual cetyl-pyridinium ions. The solution is then sterilized by filtration and sodium hyaluronate is precipitated by the addition of sterile organic solvent, e.g. sterile ethanol. The sodium hyaluronate so produced can be air dried under sterile conditions.

The sodium hyaluronate is suitable for use in compositions of cosmetic grade and clinical grade sodium hyaluronate and other suitable carriers, e.g. glycerol, polypropylene glycol, sorbitol, collagen, polyethylene glycol.

The cosmetic grade composition of sodium hyaluronate produced by the methods of this invention is characterized by an absence of skin irritation. It contains between about 87% and 91% sodium hyaluronate of a molecular weight between about 700,000 and 1,500,000 daltons and a ratio of glucoronic acid to N-acetyl glucosamine of 1:1, from about 8% to about 12% by weight water, from about 4% to about 5% by weight sodi-

$3.5x10^6$ daltons and of different grades of purity have also been produced by the methods of this invention.

The vitreous test in the Owl Monkey Eye was performed essentially as described in U.S. Patent No. 4,141,973 of E.A. Balazs (1979).

The invention also concerns the microorganism Streptococcus zooepidemicus HA-116 ATCC No. 39920 or mutants derived therefrom. This microorganism was derived by a method of selecting microorganisms which produce an enhanced amount of hyaluronic acid and which lack hemolytic activity. The method comprises treating microorganisms that produce hyaluronic acid, such as microorganisms of the genus Streptococcus, with a suitable mutagen capable of producing mutants of the organism, e.g. nitrosoguanidine. The mutants are grown on a suitable solid medium, e.g. Todd-Hewit agar, and mucoid colonies are identified. These colonies are recovered from the solid medium and grown on blood agar. The colonies which do not lyse hemoglobin are then selected and used for the production of hyaluronic acid in accordance with the methods of this invention.

a strain of *S. zooepidemicus*. *Streptococcus zooepdimicus* HA-116 has been deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Md 20852, pursuant to the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purpose of Patent Procedure, and has been assigned accession number ATCC 39920.

THE FERMENTATION PROCESS

In addition to the use of the selected mutant HA-116, we have devised several other unique procedures to increase the yields and the molecular weight of the hyaluronic acid produced by bacterial fermentation and to shorten fermentation time. This includes (i) maintenance of high levels of magnesium ion concentrations; and (ii) performance of aerobic fermentations with a high rate of aeration and vigorous agitation.

In a preferred embodiment of the invention the composition of the fermentation medium is as follows:

Component	Concentration (grams/liter)
Casein hydrolysate	20
Yeast extract	10
NaCl	2
$MgSO_4.7H_2O$	1.5
K_2HPO_4	2.5
Glucose	5

In a more preferred embodiment of the invention, the concentration in the fermentation medium of $MgSO_4.7H_2O$ is 1.0g/1, the concentration of glucose is 10g/1 and the other components have the same concentrations as above.

The pH of the medium is maintained at about 7.0 by continuous addition of 5N NaOH upon demand of a pH

Stage A

This stage comprises the removal of the bacteria and other insoluble materials by filtration, followed by three successive sedimentations by isopropanol and treatment with activated charcoal.

When only cosmetic grade material is prepared, the fermentation broth is heated for 20 minutes at a temperature of about 90°C and at a pH of about 5.0 prior to filtration. At this time no dilution is necessary. For the preparation of a clinical-grade high molecular weight material, the fermentation broth is cooled with ice to a temperature from about 10° to about 15°C, diluted 3- to 4-fold with 3% sodium acetate, adjusted to a pH of about 7.0 and then subjected to filtration. Diatomaceous-earth type filter-aid, e.g. .5 grams/liter of Celatom FW-14, Eagle-Picker Industries, Inc., Cincinnati, Ohio, is used in conjunction with a vacuum-type or pressure filter. Sodium hyaluronate is precipitated from the filtrate by addition of 1 volume of isopropanol. The precipitate is redissolved in an equal volume of 3% sodium acetate, and the material precipitated again with isopropanol. The second precipitate is redissolved in 3% sodium acetate, then 1 gram/liter of activated charcoal is added and the mixture is stirred for about 1 hour. This suspension is filtered and the sodium hyaluronate is precipitated by addition of isopropanol, washed with isopropanol and finally ground and air-dried to give a "cosmetic-grade" product.

Stage B

Cosmetic-grade sodium hayluronate is purified by two successive precipitations of its cetyl-pyridinium salt, followed by adsorption of impurities on a magnesium

PURIFICATION PROCEDURES II and III

Alternatively, two independent purification methods may be employed to obtain cosmetic grade and clinical grade sodium hyaluronate. These procedures are preferred procedures for obtaining sodium hyaluronate. Procedure II yields a low molecular weight "cosmetic grade" sodium hyaluronate, and Procedure III yields a high purity, high molecular weight non-inflammatory sodium hyaluronate suitable for clinical application.

Procedure II

At the conclusion of fermentation, the fermentation broth is heated to about 90°C, then the pH is adjusted to about 5.0 and the medium kept at 80° for 40 minutes. This step is terminated by adjusting the pH to 7.0 and cooling to about 20°C. This heating process brings about a drop in the molecular weight of the hyaluronate to about $1\text{-}1.5 \times 10^6$ dalton.

The sodium hyaluronate is precipitated from the fermentation mixture by addition of 1.5 volumes of ethanol. The precipitate is further washed with ethanol to eliminate a large portion of the microorganisms. This crude material is redissolved in aqueous 3% sodium acetate containing 0.1% parahydroxybenzoic acid methyl ester. The volume is adjusted to give about 2-3 grams per liter of hyaluronate. One gram per liter of activated charcoal and 40 grams per liter of a diatomaeous earth-type filter-aid, e.g. Celatom FW-1, Eagle-Picker Industries, Inc., Cincinnati, Ohio, are added to the solution and stirred for at least 1 hour. The mixture is then filtered through a filter-aid cake. Sodium hyaluronate is precipitated by addition of 1.5 volumes of ethanol and the precipitate is redissolved in an

Florisil Adsorption

A solution of about 0.1-0.15% sodium hyaluronate in sterile pyrogen-free 1M NaCl is passed through a column of 30-60 mesh activated Florisil e.g. 20 gr Florisil per liter of solution. The solution is then rendered germ-free by filtration through a 0.2 um filter. Sodium hyaluronate is precipitated by ethanol (1.5 volumes), followed by washing with analytical grade ethanol. The precipitate is finally dried by a stream of sterile nitrogen.

The yield of hyaluronic acid in this procedure is about 70-80%.

PROPERTIES OF THE PRODUCT SODIUM HYALURONATE

Sodium Hyaluronate Grade I

Sodium hyaluronate grade I is "cosmetic grade" sodium hyaluronate that is obtained after purification Stage A of Procedure I or Procedure II. Its properties are as described below:

a. Content of Sodium Hyaluronate: 87-91%, assayed by the modified carbazole method, Bitter and Muir, Anal. Biochem. 4, 330 (1962) using Sigma hyaluronic acid Type I, cat. # H 1751, as a reference standard.

b. Average Molecular Weight: From about 700,000 to about 1,500,000 daltons, calculated from the limiting viscosity number esentially as described by Laurent et al., Biochem. Biophys. Acta 42, 476 (1960). A representative calculation of intrinsic viscosity and molecular weight is shown below.

C - concentration in gr/ml

(η) - intrinsic viscosity (limiting viscosity number)

$$(\eta) = \lim_{C \to 0} \eta_{sp}/C$$

Determination of intrinsic viscosity (η):

The viscosity of a 0.1% sodium hyaluronate solution and of two fold, three fold and four fold dilutions of this solution were measured. The concentration of sodium hyaluronate was determined by the carbozole method. sp/C was plotted versus C and extrapolated linearly to C=0. (η) was obtained from the intersect of the line with the Y-axis.

Determination of molecular weight:

The molecular weight of sodium hyaluronate was calculated from the empirically-established Mark-Houwink relationship

$$(\eta) = 0.0403 \cdot M^{0.775}$$

wherein M is the molecular weight in daltons. The above relationship was used to determine the molecular weight of various lots of NaHA produced. The relationship is shown in Table I.

i. Absence of Skin Irritation: This is determined for a 1% solution by (i) Draize dermal irritation test in rabbits, Draize, J.H., in: "Appraisal of the Safety of Chemicals in Food, Drugs and Cosmetics". Association of Food and Drug Officials of the United States, Austin, Texas, pp. 46-49 (1959); (ii) Delayed contact hypersensitivity test in Guinea pigs, Magnusson and Kligman, J. Invest. Dermatol. 52, 268 (1969).

Sodium Hyaluronate Grade II

Sodium hyaluronate Grade II is "clinical grade" sodium hyaluronate obtained after purification through Stage B of Procedure I or after purification by Procedure III. Its properties are as described below:

a. Content of Sodium Hyaluronate: 88-92%.

b. Average Molecular Weight: More than $7x10^5$ daltons, usually in the range from about 2 to about $3.5x10^6$ daltons for NaHA purified through Stage B of Procedure I and in the range from about 2 to about $4.4x10^6$ daltons for NaHA purified by Procedure III. These molecular weight ranges are calculated from the limiting viscosity number as described above.

c. Ratio of Glucuronic Acid/N-acetyl Glucosamine: 1/1.

d. Water Content: 10% ± 2%

e. Protein: Undetectable (less than 0.01%).

f. Sodium Ions: 5% ± 1%

cells/ml, and 0.25 ml portions are taken for 90 min incubation with 0.5 ml of 2 mg/ml cytochrome C and graded amounts (0, 2, 10, and 20 mg final) of phorbol myristate acetate (PMA). PMA is an activator of the oxidative "burst" system. The media are centrifuged at 1,500 RPM for 15 min and the absorbance of the supernatants is determined at 550 nm.

Inflammation is indicated by an increase in both the number of peritoneal cells and the maximal ability to respond to PMA and reduce the cytochrome C. Hence, an index of inflammation is defined as the activity (in nmoles of superoxide radicals formed) of all the white cells obtained from one mouse. A sample is regarded as non-inflammatory if the inflammation index is not significantly higher than that obtained from mice injected with saline alone.

component	concentration
Casein hydrolysate	about 10-30
Yeast extract	about 5-15
NaCl	about 2
$MgSO_4 \cdot 7H_2O$	above about 0.5
K_2HPO_4	about 2.5
Glucose	about 2-15

7. The method of claim 2, wherein recovering the sodium hyaluronate comprises treating the medium containing the microorganisms so as to remove the microorganism and other materials insoluble in the medium, precipitating the sodium hyaluronate from the medium and then recovering the precipitate.

8. The method of claim 7 further comprising grinding and then drying the precipitate.

9. The method of claim 7, further comprising adjusting the pH of the medium containing the microorganism to about 5.0 and then heating the medium for a suitable period of time at a temperature between about 80° and 95°C prior to treating the medium to remove the microorganism.

10. The method of claim 9, wherein the medium is heated for about 20 minutes at about 90°C.

11. The method of claim 9, wherein the medium is heated for about 40 minutes at 80°C.

12. The method of claim 7, wherein the treating comprises filtration.

19. Th m thod of claim 14, further comprising adjusting the pH of the medium containing the microorganism to about 7.0, cooling the medium to a temperature between about 4° and 15°C and then diluting the medium with 3% aqueous sodium acetate prior to treating the medium to remove the microorganism.

20. The method of claim 19, further comprising redissolving the precipitate in 0.15M aqueous NaCl, adding cetyl-pyridinium chloride to form the cetyl-pyridinium salt of hyaluronic acid, dissolving the cetyl-pyridinium salt in aqueous NaCl (at least about 1M) and ethanol, adding organic solvent and recovering the sodium hyaluronate.

21. The method of claim 20, further comprising redissolving the recovered sodium hyaluronate in 0.15M aqueous NaCl, adding cetyl-pyridinium chloride to again form the cetyl-pyridinium salt of hyaluronic acid, dissolving the cetyl-pyridinium salt in aqueous NaCl (at least about 1M) and 10% ethanol, precipitating the sodium hyaluronate with an organic solvent, dissolving the sodium salt in NaCl solution, contacting the resulting solution with magnesium silicate absorbent to remove impurities and residual cetyl-pyridinium ions, sterilizing the solution and adding sterile organic solvent to precipitate the sodium hyaluronate from the solution.

22. The method of claim 21, wherein the organic solvent is isopropanol.

23. Th method of claim 21, further comprising air-drying the sodium hyaluronate precipitate under sterile conditions.

31. The method of claim 30, wh rein each of the first, second and third organic solvents are isopropanol, ethanol or acetone.

32. The method of claim 30, wherein the first, second and third organic solvents are ethanol.

33. The method of claim 30, further comprising grinding and then drying the precipitate of the third organic solvent.

34. The method of claims 25, 26 or 30, further comprising adjusting the pH of the medium containing the microorganism to about 7.0 and cooling the medium to a temperature between about 4° and 20°C prior to adding the first organic solvent.

35. The method of claim 25, wherein the suitable aqueous solution is 0.15M aqueous NaCl solution containing 0.1% parahydroxybenzoic acid methyl ester.

36. The method of claim 35, further comprising adding cetyl-pyridinium chloride in 0.15M NaCl to the hyaluronate solution to form the cetyl-pyridinium salt of hyaluronic acid, redissolving the cetyl-pyridinum salt in aqueous NaCl (at least about 1M) containing 10% ethanol and adding organic solvent to recover the sodium hyaluronate.

37. The method of claim 36, further comprising redissolving the recovered sodium hyaluronate in 0.15M aqueous NaCl, adding cetyl-pyridinium chloride to again form the cetyl-pyridinium salt of hyaluronic acid, redissolving the cetyl-pyridinium salt in aqueous NaCl (at least about 1M) containing 10% ethanol, adding

12% by weight water, from about 4% to about 6% by weight sodium ion, less than 0.01% by weight protein, less than 0.001% by weight sulfate, less than 0.02% by weight nucleic acid and less than 0.2% by weight neutral sugar.

43. A composition of sodium hyaluronate characterized by an absence of pyrogenicity and inflammatory activity comprising between about 88% and 92% by weight sodium hyaluronate of an average molecular weight from about 2 to about 4.4×10^6 daltons and a glucoronic acid to N-acetyl glucosamine ratio of 1:1, from about 8% to about 12% by weight water, from about 4% to about 6% by weight sodium ion, less than 0.01% by weight protein, less than 0.001% by weight sulfate, less than 0.02% by weight nucleic acid and less than 0.2% by weight neutral sugar.

44. A composition containing the sodium hyaluronate of claim 41, 42 or 43 and a suitable carrier.

45. A composition of high molecular weight sodium hyaluronate characterized by a minimum viscosity of about 3.5 m^3/kg, a minimum average molecular weight of about 3.5×10^6 daltons, a specific optical rotation measured at 25°C and at a wavelength of 436nm from about 155° to 165°, a protein content of less than about 1 mg/gram, an absorbance at the wavelength of 257 nm of less than about 0.5, endotoxin of less than about 0.05 ng/ml., less than about 0.2 mg/g of iron, less than about 0.2 mg/g of copper, an infiltration of less than about 200 white blood cells per mm^3 of aqueous humor of owl monkey eye when one ml. of a 1% solution of the composition dissolved in physiological buffer

INTERNATIONAL SEARCH REPORT

International Application No PCT/US86/00066

I. CLASSIFICATI N OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

INT. Cl.[4] C12P 19/04, C08B 37/00, A61K 31/715

U.S. Cl. 435/101 536/55.1 514/54

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
U.S.	435/101, 253, 801, 803, 818, 885 536/55.1, 123 514/54, 62, 847, 915

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]

COMPUTER DATA BASES: CHEM. ABSTRACTS FILE 308, 309, 310, 311, 320; BIOSIS FILE 5, 55, 255

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
Y	US, A, 4,141,973 published 27 February 1979	40,42-47
Y	US, A, 4,303,676 published 01 December 1981	40
Y,P	US, A, 4,517,295 published 14 May 1985	2,6,7,12, 14-16,25, 40
Y	JA, A, 0,037,001 published 04 March 1983	9-11,26, 27
Y,P	EP, A, 0,143,393 published 05 June 1985	2,7,12, 14-16,20-22,25,31, 36-38,40 42-47
Y	N, Veterinary Microbiology, issued 1982, Vol. 7, No. 1, A.M. Buchanan et al., 'Recovery of Microorganisms from Synoxial and Pleural Fluids of Animals Using Hyperosmolar Media', see pages 19-33.	1,3,4

* Special categories of cited documents: [15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATI N

Date of the Actual Completion of the International Search [2]	Date of Mailing of this International Search Report [2]
03 April 1986	08 APR 1986
International Searching Authority [1]	Signature of Authorized Officer [10]
ISA/US	Elizabeth C. Weimar Elizabeth C. Weimar